Molecular Identification of Two Closely Related Species of Mealybugs of the Genus *Planococcus* (Homoptera: Pseudococcidae)

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ABSTRACT Morphological identification of the mealybug species *Planococcus citri* (Risso) and *P. minor* (Maskell), two serious agricultural pests, is often complicated by the existence of intermediate forms and a lack of knowledge of the intraspecific variation that occurs in each species. In this study, we have explored the utility of two molecular markers, the mitochondrial gene cytochrome oxidase I (COI) and the nuclear gene, elongation factor 1α (EF-1α) to ascertain the identity of these species and to provide reliable characters for their identification. Results from maximum parsimony analysis of DNA sequence data from both genes indicate the existence of a third clade from the Hawaiian Islands, whose members are distinct from both *P. citri* and *P. minor*. The individuals that group in this additional clade, although morphologically identical to *P. citri*, cluster with *P. minor* in ≈50% of the cladograms obtained with the COI, and 80% of the cladograms obtained with EF-1α. Our studies show that COI, in combination with morphological and geographical data, can be used to accurately identify the *P. minor* clade, the *P. citri* clade, and the clade from the Hawaiian Islands in most cases. Given a few instances in which identification resulting from COI and EF-1α were in conflict, however, our results must be interpreted with caution and until additional studies are performed, no changes are proposed in the taxonomy of this species complex.

KEY WORDS Pseudococcidae, *Planococcus*, taxonomy, cytochrome oxidase I, elongation factor 1α

Identification of many mealybug species in the genus *Planococcus* has been difficult at best (Cox and Wetton 1988). Research by biological control specialists has shown that certain chalcidoid wasps can discriminate seemingly identical species of *Planococcus* (Rosen and DeBach 1977) and differences in pheromone structure have substantiated the existence of cryptic species (Rotundo and Tremblay 1975). If only species identification was as easy for mealybug as it is for parasitoids!

The first definitive treatment of *Planococcus* was by Ezzat and McConnell (1956), and it included 19 species. In this contribution, species were separated by characters such as the length of certain setae, the number of tubular ducts, the presence of translucent pores on the hind legs, and the distribution of multilocular pores. In the early 1980s, Jennifer Cox became interested in the unusual amount of morphological variation that she found in certain species of the genus, and she undertook a study that demonstrated that environmental conditions could affect morphological characters often used to identify species (Cox 1983). By rearing the first instar progeny (crawlers) of a single female on the same host but under different temperature regimes, she showed that higher temperatures induced smaller specimens, fewer pores, shorter appendages, and shorter setae. *Planococcus citricus* Ezzat & McConnell was shown to be a warm temperature variant of *Planococcus citri* (Risso) (Cox 1981). The greatest number of tubular ducts was present on adult females reared at optimal intermediate temperatures, and the smallest number was present in adult females reared at temperatures higher and lower than the optimal temperature (Cox 1983). By rearing specimens under various controlled environmental conditions, Cox was able to quantify the amount of phenotypic plasticity within a species and was better able to find useful diagnostic characters. Cox (1989) revised *Planococcus* and included 35 species, several of which could only be reliably identified using a suite of highly variable characters. For example, because of the variation in the diagnostic characters used to distinguish individuals of *Planococcus minor* (Maskell) and *P. citri*, she developed a matrix of six characters to be scored using a point system, often referred to as the "Cox score." Specimens having a total score of 35 or less were determined to be *P. minor*, and those having a total score >35 were determined to be *P. citri*.

In research conducted by Cox and Wetton (1988), no single diagnostic feature could be used to distinguish *Planococcus ficus* (Signoret) from *P. halli* Ezzat

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Table 1. Collection details of *Planococcus* species and outgroup taxa used in the analyses (GenBank sequences not listed)

Label	Country	Locality, date, and collector	Host
MB45	American Samoa	Mepusage, Tutuila, 4 July 2006, M. Schaedick	*Clerodendrum paniculatum* L.
MB12	Australia	Queensland, 21 Aug. 2005	*Morinda citrifolia* L.
MB65	Brazil	Espirito Santo, Linhares, 21 Feb. 2005, D. Martins	Biden pilosa root
MB59	Brazil	Espirito Santo, Vitória, 10 Dec. 2004, M. Culik	No information
MB60	Brazil	Espirito Santo, Linhares, 16 Dec. 2006	No information
MB34	Ecuador	25 April 2006, C. Gaona	No information
MB27, MB28	France	Alpes Maritimes, 6 Dec. 2005, Menton	*Citrus aurantium* L.
MB58	Indonesia	26 June 2006	"Ornamental plants"
MB5, 6	Israel	5 June 2005	*Rumex acetosa* L.
MB61	Mexico	2 Jan. 2006, D. Martha	*Punica granatum* L.
MB20	Nigeria	25 Aug. 2005, M. Gilley	*Dioscorea* sp
MB56	South Africa	Stellenbosh, 2006	
MB37	South Korea	7 Dec. 2005, Jeju Kor	No information
MB62	Taiwan	No further information	Lab pumpkin
MB14	Thailand	Thani Province, 17 June 2004, P. S. Cranston	Ranbutan fruit
MB38, MB40	Trinidad and Tobago	Centeno, 10 July 2006, A.Francis	No information
MB50	Trinidad and Tobago	St. Augustine, UWI campus, 28 July 2006, A. Francis	Cacao
MB42	United Kingdom	Wye Bugs, Imperial College, 2006, Kent	No information
MB13	United States	CA, 3 Oct. 2001, L. Marin	No information
MB9	United States	CA, Davis, 13 July 2001, S. K. Kelley	*Citrus sinensis* (L.)
MB57	United States	CA, Pittsburg, Wallmart, 18 Aug. 2006, R. Fonseca	No information
MB17	United States	CA, San Joaquim, 12 July 2004, F. D. Minozzoli	No information
MB10	United States	CA, Riverside, 18 May 2005, M. Lanthi	No information
MB32, 33, 35	United States	CA, San Luiz Obispo 7 June, 2006, C. Darling	*Radermachera sinica* (Hance)
MB43	United States	FL	No information
MB47	United States	FL, Citrus Co. July 2006, G. Hodges	C. paniculatum
MB48	United States	FL, Orange Co., Apoka, July 2006, G. Hodges	No information
MB30	United States	FL, Orange Co., Apoka, 5 Jan. 2006, L. Osborne	Potato
DMPS27	United States	Hawaii, Pali, Oahu, 25 April 2005, J. Yalemar	*Pipturus* sp.
MB44	United States	Hawaii	No information
MB49	United States	Hawaii	No information
MB1, MB4	Viet Nam	No further information	*Nephelium lappaceum* L.
DMPS38	West Indies	St Kitts, Bird Rock	No information

& McConnell. Multivariate analysis was required to demonstrate the existence of the two taxa. Cox and Wetton were able to demonstrate that the translucent pores, considered by some to be diagnostic for *P. ficus*, are present in most individuals reared at high temperatures and are absent in most individuals reared at low temperatures. The conclusion of Cox and Wetton (1988) exemplifies the difficulties of properly identifying species of *Planococcus:* "Whether or not these two forms are really distinct biological entities might be investigated at some time in the future. However, because two names are available, it seems most appropriate to regard them as separate species."

Here, we have pursued the suggestion of Cox and Wetton (1988) to investigate alternative character systems that can be used to distinguish cryptic species of *Planococcus.* For this purpose, we have used sequences of two genes, the mitochondrial gene cytochrome oxidase I (COI) and the nuclear gene elongation factor 1α (EF-1α). We have focused on *P. citri* and *P. minor* because of the difficulty of determining the identity of intermediate forms based on the Cox scoring system and for the special significance of these two species to U.S. agriculture and regulatory efforts. *P. citri* is abundant and a serious pest in the United States; *P. minor*, although frequently intercepted at U.S. ports-of-entry, has never been confirmed as occurring in the United States (Ben-Dov et al. 2006).

Materials and Methods

Molecular Methods. Adult females of *Planococcus* obtained from different hosts in various locations around the world (Table 1) were collected and preserved in 95%–100% ethanol for various amounts of time. In the laboratory, specimens were transferred to vials containing 100% ethanol and stored in a −80°C freezer until needed for DNA extraction.

DNA was generally extracted from one specimen only, following the Blood and Body Fluid Protocol of the QIAamp blood kit (QIAGEN, Valencia, CA), with the following modification: specimens were not ground but rather punched with a sterilized needle on the side. After adding proteinase K, specimens were left in an incubator at 70°C for an average of 24 h. During the DNA extraction procedure, these specimens were retrieved, then mounted and kept as vouchers. They are deposited at the National Museum of Natural History collection.

Polymerase chain reaction (PCR) was carried out in a Stratagene Robocycler (Stratagene, La Jolla, CA). PCR was performed with TaKaRa Ex *Taq* (Takahara Bio USA, Madison, WI) following the manufacturer's protocol with 4 μl of DNA in a total of 50 μl of solution. The following universal primers (Simon et al. 1994) were used to amplify ≈900 bp of the COI, spanning from the 3′ half of the COI gene to the leucine transfer RNA (LEU tRNA): CJ-J-2183 (alias Jerry, 5′-CAACATTTATTTTGATTTTT-

TGGN-3′) and TL2-N-3014 (alias Pat, 5′-TCCATTGCACTAATCTGCCATATTA-3′). Because some samples failed to amplify with the last primer, we designed a *P. citri*/*P. minor* specific reverse primer (herein called 3014.3R: 5′-GTATGATTTAAATTAGGTGA-3′) that flanks the 3′ end of the COI. For amplification of the EF-1α, we used the primers EF-40 (5′-GTC GTG ATC GGA CAG GTC GAT TCC GG-3′) and EF-53 (5′-ATGTGAGCATRTGRCAATCCAA-3′) (Scheffer and Lewis 2001), obtaining amplicons of ≈1,550 bp. The same program protocol was used to obtain products from both genes: 94°C for 4 min followed by 30 to 50 cycles of 94°C for 1 min, 48–56°C for 1 min, and 72°C for 1 min 30 s with a final extension at 72°C for 4 min. PCR product was purified using the QIAquick PCR purification kits (QIAGEN). Sequencing reactions were carried out with Big Dye Terminator sequencing kits (Applied Biosystems, Foster City, CA). Primers for sequencing the COI were the same as used for amplification. For the EF-1α, the following primers were used: forward primers, MB-EF1 (5′-GGACATTTAATYTACAAATGCGG-3′) and EF-90 (5′-AASATGCCNTGGTTCAAGGGATGG-3′); and reverse primers, EF1–61 (5′-GCA GCT GGA TTG TAA CCG ATC TTC-3′) and EF-53.R (5′ ATGTGAGCATRTGRCAATCCAA 3′). The first three primers (MB-EF1, EF-90, and EF1–61) were designed in Sonja Scheffer's lab (USDA–BARC) and have not been previously published. Sequence data were obtained by analyzing samples on an ABI-377 Automated DNA sequencer (Applied Biosystems).

Sequences from both strands were assembled and edited if necessary using Sequencher (Gene Codes, Ann Arbor, MI). Multiple alignments were carried out with CLUSTAL X, version 1.81 (Thompson et al. 1997). Each haplotype (Table 3) was represented only once in the analysis. Several of the *P. citri* mitochondrial sequences had a deletion that interrupts the reading frame, leaving a gap at position 140 of the alignment. Sequences with and without this deletion were treated as different haplotypes, even when they were otherwise identical. All sequences were deposited in GenBank under accession numbers EU250490–EU250516 (EF-1α) and EU250517–EU250574 (COI).

Phylogenetic Analysis. Mega 2.0 (Kumar et al. 2001) was used to calculate overall sequence divergences (based on uncorrected distances). Gapped regions and regions where the alignment was ambiguous (EF-1α) were excluded from all analyses. Additionally, a stretch of coding region spanning 45 bp (between positions 855–950) generated poor sequences for seven taxa in the EF-1α data set. This region was also excluded from the analysis (including this region did not change the results but dramatically increased the number of shortest trees). Maximum parsimony (MP) was used to estimate trees with PAUP*, version 4.0b10 (Swofford 2003). Tree search was heuristic, by using tree-bisection-reconnection branch swapping with 100 random taxon addition sequences. Support for clades was estimated by bootstrapping (500 replications) by using the same heuristic settings as mentioned above. Trees were rooted using two other species of *Planococcus*, *P. ficus*, and *P. halli*. Each data set (COI and EF-1α) was analyzed separately.

Table 2. Mean genetic distances (uncorrected distances) between the three clades represented in the cladograms of Figs. 1 and 2, calculated from the COI (left, below) and EF-1α (right, above)

	Hawaiian clade	*P. minor* clade	*P. citri* clade
Hawaiian clade	—	0.6	0.9
P. minor clade	1.93	—	0.9
P. citri clade	1.98	1.9	—

Distances given in percentage.

Results

The shortest trees resulting from the two different data sets (COI and EF-1α) are summarized in Figs. 1 and 2. The molecular data indicate that there are three distinct clades within the *P. citri–P. minor* species complex: the *P. citri* and the *P. minor* clades, encompassing specimens from various locations around the world, and the "Hawaiian clade," with specimens from the Hawaiian Islands only. This result differs from previous morphological studies that acknowledge only two distinct clades, or "species," within this complex (see Introduction). With a few exceptions, discussed in the remaining sections, tree topologies resulting from the COI data set agree with tree topologies resulting from the EF-1α data set. Below we present the results for each data set (COI and EF-1α) separately.

COI. The COI alignment with each haplotype represented only once was unambiguous. It totaled 23 sequences with 700 aligned sites. Out of the 612 conserved sites, 88 were variable, 54 were parsimony-informative and 34 were singletons. Typical base composition was highly A-T rich, as it is typical for insects (80.3%) (A = 34.6%, T = 45.7%, C = 10.3%, G = 9.5%). The transition/transversion ratio was 4.05.

About half of the trees obtained with maximum parsimony (49%) placed the Hawaiian clade as the sister-group of *P. minor* (MP bootstrap = 99%) + *P. citri* (MP bootstrap = 97%), but the relationship between these three groups was unresolved (Fig. 1) in the *strict* consensus cladogram of the 26,820 shortest trees (108 steps, consistency index [CI] = 0.78, retention index [RI] = 0.9). Genetic distances among individuals within the *P. citri* clade, represented by 12 haplotypes in the COI matrix, ranged from 0 to 0.61%. Genetic distances among the *P. minor* clade, represented by seven haplotypes, ranged from 0 to 0.6%. Average pairwise distances between the three clades were slightly <2% (Table 2).

Two haplotypes within the *P. citri* clade were represented by more than one sequence: COI.H1 (N = 13) and COI.H2 (N = 16). Within the *P. minor* clade, four haplotypes were represented by more than one sequence: COI.H3 (N = 2), COI.H4 (N = 3), COI.H5 (N = 2), and COI.H7 (N = 4) (Table 3). Identical sequences often originated from different localities and/or countries, with two exceptions: COI.H2, a haplotype that clustered with the *P. citri* clade, contains

Table 3. COX scores for the COI and EF-1α haplotypes found within the *P. citri*/*P. minor* species complex

Cox score (voucher)	Label	Country	COI ID	COI haplotype	EF1 ID	EF1 haplotype
5	MB62	Taiwan	M	COI.H7	M	EF1.H3
	MB40	Trinidad and Tobago	M	COI.H4	X	X
	MB40.03		M	COI.H4	M	EF1.H5
	MB50		M	COI.H8	M	EF1.H4
	MB1	Viet Nam	M	COI.H7	M	EF1.H3
10	MB12 (12,15)	Australia	M	COI.H3	M	EF1.H3
	MB45.1	American Samoa	M	COI.H7	X	X
	MB34	**Ecuador**	**M**	**COI.H17**	**C**	**EF1.H7**
	MB56 (20)	South Africa	C	COI.H19	C	EF1.H17
	MB4	Viet Nam	M	COI.H5	M	EF1.H3
15	MB45.3	American Samoa	M	COI.H7	X	X
	MB65	Brazil	M	COI.H10	M	EF1.H3
	MB35.9	United States, Calfornia	C	COI.H2	X	X
20	**MB58**	**Indonesia**	**M**	**COI.H3**	**C**	**EF1.H1**
25	MB32.3	United States, Calfornia	C	COI.H2	X	X
30	MB35.10	United States, Calfornia	C	COI.H2	X	X
	MB30	United States, Florida	C	COI.H2	X	X
	MB38 (15)	Trinidad and Tobago	M	COI.H4	X	X
35	MB17	United States, Calfornia	C	COI.H1	C	EF1.H1
	MB33.5	United States, Calfornia	X	X	C	EF1.H9
	MB35.1	United States, Calfornia	C	COI.H1	C	EF1.H12
	MB35.14	United States, Calfornia	C	COI.H2	X	X
	MB35.17	United States, Calfornia	C	COI.H2	X	X
40	MB27	France	C	COI.H1	C	EF1.H14
	MB.35.6	United States, Calfornia	C	COI.H14	X	EF1.H11
	MB.35.8	United States, Calfornia	C	COI.H2	X	X
45	MB33.7	United States, Calfornia	C	COI.H1	X	X
	MB35.4	United States, Calfornia	C	COI.H1	X	X
35–45	MB32.6	United States, Calfornia	C	COI.H2	X	X
	MB35.16	United States, Calfornia	C	COI.H2	X	X
	MB32.4	United States, Calfornia	C	COI.H1	X	X
50	MB32.2	United States, Calfornia	C	COI.H2	X	X
60	MB10	United States, Calfornia	C	COI.H2	C	EF1.H1
	MB48	United States, Florida	C	COI.H2	X	EF1.H1
	MB47	United States, Florida	C	COI.H16	X	EF1.H10
	MB43	United States, Florida	C	COI.H9	X	X
	MB44.1	United States, Hawaii	H	COI.H6	H	EF1.H2
70	MB60	Brazil	C	COI.H13	C	EF1.H18
	MB5	Israel	C	COI.H11	C	EF1.H15
	MB37	South Korea	C	COI.H1	X	X
	MB44.4	United States, Hawaii	C	COI.H15	X	X
55–70	MB33.3	United States, Calfornia	C	COI.H2	X	X
75	MB59	Brazil	C	COI.H1	M	EF1.H3
	MB35.2	United States, Calfornia	C	COI.H2	X	X
	MB44.2	United States, Hawaii	H	COI.H6	X	X
80	Dmps27 (75*4)	United States, Hawaii	C	COI.H1	H	EF1.H6
85	MB33.6	United States, Calfornia	C	COI.H2	X	X
100	MB44.3	United States, Hawaii	H	COI.H6	X	X
	MB42 (30)	United Kingdom	C	COI.H1	C	EF1.H16
NS	MB28	France	C	COI.H18	X	X
	MB6	Israel	C	COI.H12	X	X
	MB61	Mexico	X	X	C	EF1.H8
	DQ238221.1	South Africa	C	COI.H19	X	X
	DMPS20	South Korea	C	COI.H1	X	X
	MB14	Thailand	M	COI.H5	M	EF1.H3
	MB9	United States, Calfornia	C	COI.H1	C	EF1.H1
	MB57	United States, Calfornia	C	COI.H2	C	EF1.H13
	AF483204.1	United States, Florida	C	X	X	X
	MB49.1	United States, Hawaii	H	COI.H6	H	EF1.H2
	DMPS38	West Indies	C	COI.H1	X	X

Cox scores given within parentheses after the specimen label were calculated from other specimens present in the same infestation as the voucher specimen. Specimens whose EF-1α and COI IDs conflict are in bold. Abbreviations: C, *P. citri;* M, *P. minor;* H, Hawaiian clade; NS, no score; and X, specimen not sequenced for the given gene.

only specimens collected in California and Florida (U.S.), and COI.H4, a haplotype that clustered with the *P. minor* clade, only contains specimens collected in Trinidad and Tobago (Centeno) (Fig. 1). Four specimens (MB30, MB32.3, MB35.9, and MB35.10, all collected in the United States) whose sequences belong in the *P. citri* haplotype COI.H2 had Cox scores compatible with their identification as *P. minor*, and four specimens (MB17, MB35.1, MB35.14, and MB35.17, also collected in the United States) whose sequences belong in the *P. citri* haplotypes COI.H1 and COI.H2 could not be identified with certainty

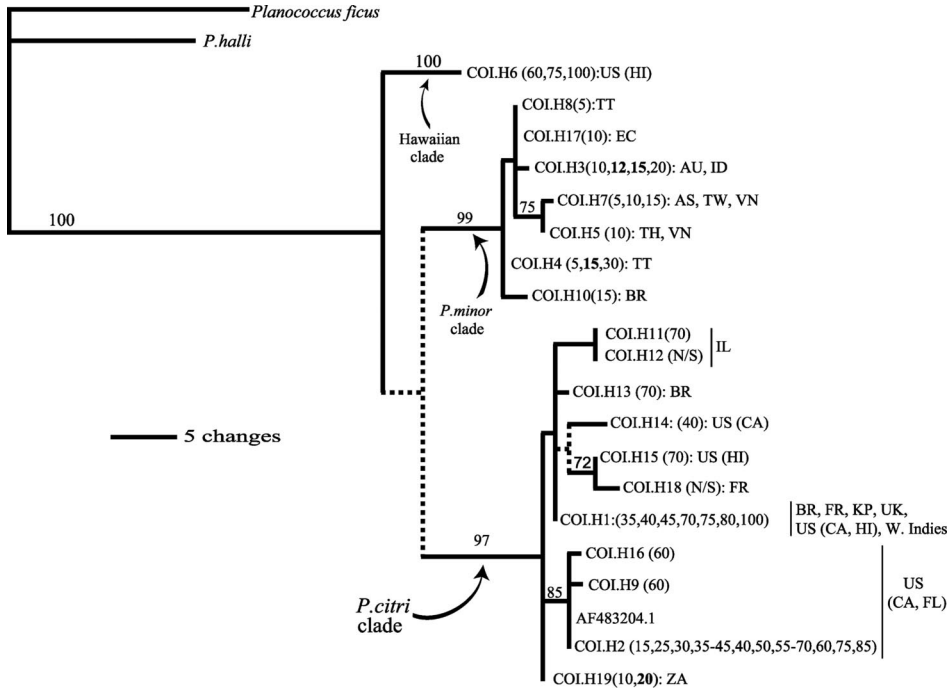

Fig. 1. Phylogram randomly chosen to represent the phylogenetic relationships of the *P. citri–P. minor* species complex obtained with maximum parsimony of the COI data set (108 steps, CI = 0.78, RI = 0.9). The numbers above the nodes are bootstrap values (only support measures higher than 69% are shown). Cox scores obtained from individuals of each haplotype are listed in parentheses. Boldfaced Cox scores were calculated from other specimens present in the same infestation as the voucher specimen. A dashed line indicates where the branches collapse in the strict consensus cladogram of the 26,820 shortest trees. Country abbreviations: AS, American Samoa; AU, Australia; BR, Brazil; EC, Ecuador; FR, France; ID, Indonesia; IL, Israel; KP, South Korea; TH, Thailand; TT, Trinidad and Tobago; TW, Taiwan; UK, United Kingdom; U.S., United States; VN, Viet Nam; ZA, South Africa.

based on morphology because they had borderline Cox scores (Table 3). Sequences from specimens collected in the Hawaiian Islands (composing the Hawaiian clade, COI.H6) were identical with two exceptions: DMPS27 (COI.H1, from Oahu, HI) and MB44.4 (COI.H15), both of which clustered with *P. citri* in the COI data set. Specimens collected in the Hawaiian Islands, regardless of whether they clustered with the *P. citri* or the Hawaiian clade, had Cox scores compatible with their identification as *P. citri* (Table 3).

A sequence downloaded from GenBank and originally attributed to *Dysmicoccus brevipes* (Cockerell) (AF483204.1) clustered with several sequences of *P. citri* that we have sequenced from specimens collected in the United States. Therefore, we believe that this sequence (AF483204.1) is actually *P. citri.* Another sequence originated from the same publication (Thao et al. 2002), and attributed to a specimen of *P. citri* collected in Florida (accession no. AF483206.1), is very divergent from all sequences that we have for this species; we suspect that this specimen is actually *D. brevipes.*

EF-1α. The EF-1α data set had an ambiguous region in the alignment of the ingroup sequences spanning 45 bases in the last intron (between positions1400–1435). The alignment with each haplotype represented only once totaled 20 sequences with 1,520 aligned sites. Of the 1,324 included sites, 1,133 sites were constant and 163 were variable, with 28 parsimony-informative sites. Base composition was not significantly A-T biased (58%) (T = 31.4%, C = 19.1%, A = 27.1%, and G = 22.4%), and the transition/transversion ratio was 1.27. Five introns (determined by their GT-AG splicing sites) were identified. The last intron, between positions 1385 and 1466 of the alignment, was most variable, and the alignment was ambiguous at a few sites.

Approximately 20% of the trees obtained with maximum parsimony (240 trees, 208 steps, CI = 0.76 and RI = 0.88) placed the Hawaiian clade (MP bootstrap support = 95%), represented by two different haplotypes, as the sister-group of the *P. minor* + *P. citri.* The majority of trees (80%) placed the Hawaiian clade as the sister-group of the *P. minor* clade (MP bootstrap support = 69%), represented by three different haplotypes, but the relationships between these tree clades was unresolved in the strict consensus cladogram of the 240 trees. The *P. citri* clade (MP bootstrap support = 100%) was represented by 13 different haplotypes. EF-1α results were generally congruent with the results obtained in the COI analysis (see above), with a few exceptions (Fig. 2; Table 3): in the EF-1α data set, MB59 (from Brazil) clustered with *P. minor,* being identical with other sequences in the

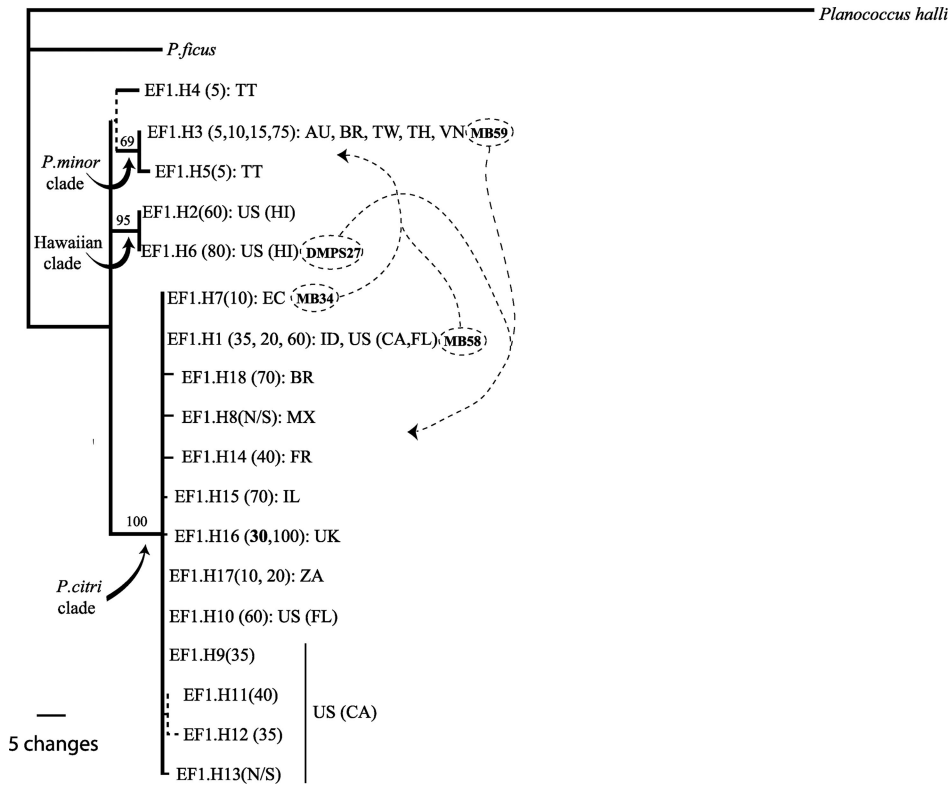

Fig. 2. Phylogram randomly chosen to represent the phylogenetic relationships of the *P. citri–P. minor* species complex obtained with maximum parsimony of the EF-1α data set (208 steps, CI = 0.76 and RI = 0.88). The numbers above the nodes are bootstrap values (only support measures 69% or higher are shown). Dashed lines indicate where branches collapse in the strict consensus cladogram of the 240 shortest trees. Cox scores obtained from individuals of each haplotype are listed in parentheses. Bold Cox scores were calculated from other specimens present in the same infestation as the voucher specimen. Label names in bold indicate exemplars that clustered with different clades in the COI analysis, and the dashed lined arrows indicate where they clustered in that analysis. Country abbreviations: AU, Australia; BR, Brazil; EC, Ecuador; FR, France; ID, Indonesia; IL, Israel; MX, Mexico; TH, Thailand; TT, Trinidad and Tobago; TW, Taiwan; UK, United Kingdom; U.S., United States; VN, Viet Nam; ZA, South Africa.

EF1.H3 haplotype. In the COI data set, MB59 clustered with the *P. citri* clade, a result more compatible with the morphological identification of the voucher. MB58 (EF1.H1, from Indonesia) and MB34 (EF1.H7, from Ecuador) both clustered with the *P. citri* clade in the EF-1α data set, contrasting with their inclusion in the *P. minor* clade according to the COI and morphological results. DMPS27 (EF1.H6, from Hawaii) joined the Hawaiian clade in the EF-1α data set. In the COI data set, this sequence clustered with the *P. citri* clade. Also contrasting with the COI results, bootstrap measures did not strongly support the placement of MB50 (EF1.H4, from Trinidad and Tobago) within the *P. minor* clade, and bootstrap support for the *P. minor* clade itself was lower than 70% in this data set (Fig. 2). Pairwise distances varied between 0 and 11.5% across the entire data set. The greatest genetic distances within the *P. minor* clade was 0.2%, but when MB50 is included in this clade (see below) it jumps to 0.6%. Within the *P. citri* clade, the longest pairwise distances were 0.3%. Sequences within the Hawaiian clade had a distance of 0%. Average distances between groups, represented in Table 2, varied from 0.6 to 0.9% (MB50 was included in the *P. minor* clade to calculate these distances).

One haplotype within *P. minor* clade, EF1.H3 (N = 5) was represented by multiple sequences from specimens collected in the Oriental, Australasian, and Neotropical regions. Within the *P. citri* clade, one haplotype (EF1.H1, N = 5) was represented by multiple sequences, most of which from specimens collected in the United States, plus one sequence from a specimen collected in Indonesia. The Hawaiian clade was represented by two haplotypes, one of which (EF1.H2, N = 2) was represented by more than one sequence, from Hawaii.

Discussion

One objective of this work was to ascertain whether morphological identification of *P. minor* and *P. citri* based on the Cox score (see Introduction for details) is congruent with clusters resulting from DNA sequence data analysis. Where there is no conflict be-

tween data sets, specimens that clustered with the *P. minor* clade had been previously identified as *P. minor* based on their morphology (Table 3; Figs. 1 and 2). However, the same is not true for *P. citri.* Of the 31 specimens with Cox scores that clustered with *P. citri* in our COI analysis, four (≈12%) were in the borderline (35) and four (≈12%) had scores compatible with their identification as *P. minor* (Table 3). These results suggest that *P. minor* individuals can almost always be identified accurately with the values used in the Cox score, but that the use of the Cox score to identify *P. citri* individuals is less reliable. One of the main agricultural and regulatory concerns for the United States is to prevent the introduction and establishment of *P. minor* into the country. Morphologically based identifications may usually suffice for this purpose, but when a *P. citri* individual is misidentified as *P. minor* because it has a low Cox score (a "false positive" identification), shipments containing agricultural products may be unnecessarily detained or excluded from entering the country, causing unnecessary economic losses.

Another opportunity for error was revealed in a case where the DNA data set disagreed with the morphologically based data set (Cox score). A specimen from Brazil (Table 3, MB59) scored high on the Cox scale, indicating that it was a *P. citri* individual; however, the specimen clustered with *P. minor* in the EF-1α data set. Even though this is the only case in our taxon sampling in which a specimen with a high Cox score clustered with *P. minor*, it is a matter of concern. Until more studies are carried out in this group, using another nuclear gene, we must take a conservative approach and consider the possibility that the EF-1α might more accurately reflect the evolutionary history of this group. If this hypothesis is correct, a COI-based identification, even in combination with morphological analysis, may fail to weed out *P. minor* individuals in a small percentage of the identifications.

Besides the discrepancies mentioned above, our analysis revealed a morphologically cryptic clade whose appearance is identical to *P. citri* but clusters with *P. minor* in 80% of the cladograms obtained with EF-1α, and in about half of the cladograms obtained with COI. The core of this clade, referred to above as the Hawaiian clade, is composed of identical sequences from the same locality in Hawaii. Nothing in particular is known about the biology, distribution and frequency of individuals of the "Hawaiian clade." As with *P. minor* and *P. citri*, there was at least one discrepancy between the two data sets in classifying individuals of this clade: one specimen (DMPS27) that clusters with the Hawaiian clade in the EF-1α data set clustered with *P. citri* in the COI data set.

The question of whether the Hawaiian clade corresponds to a new species cannot be answered with our data. Even though DNA sequences of individuals belonging in this clade are distinct from *P. citri* and *P. minor* sequences, we have adopted a conservative approach and will not be naming a new species here.

In addition to molecular and morphological data, distribution data can be used as an aid to separate *P. citri* from *P. minor*, because the range of distribution of both species does not always overlap. For example, *P. minor* seems to be absent from the Palearctic and Nearctic regions except for Mexico, where both species occur. Both species occur in the Afrotropical, Neotropical, Australasian, and Oriental regions, but even in these large regions some patchy distributions may occur. For example, *P. minor* has not been reported from Micronesia, the Hawaiian Islands, and continental Africa. Even though distribution data are less reliable to base identifications on, mostly due to unreported occurrences, insufficient geographical sampling and misleading identifications, it adds a layer of security when combined with morphology and DNA data.

This is the first published study of the molecular phylogeny of *Planococcus* species. For this reason, we do not know whether the level of COI divergence between our three clades fall within the range of that between other closely related *Planococcus* species.

Based on our data, we can predict that COI and morphology combined with locality data will result, most of the time, in an accurate identification. However, more studies are needed to sort out the conflicting information given by the two sources of DNA sequence data, and to confirm the identity of the Hawaiian clade.

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References Cited

Ben-Dov, Y., D. R. Miller, and G.A.P Gibson. 2006. ScaleNet/ *Planococcus minor*. (http://www.sel.barc.usda.gov/catalogs/pseudoco/Planococcusminor.htm).

Cox, J. M. 1981. Identification of *Planococcus citri* (Homoptera: Pseudococcidae) and the description of a new species. Syst. Entomol. 6: 47–53.

Cox, J. M. 1983. An experimental study of morphological variation in mealybugs (Homoptera: Coccoidea: Pseudococcidae). Syst. Entomol. 8: 361–382.

Cox, J. M. 1989. The mealybug genus *Planococcus* (Homoptera: Pseudococcidae). Bull. Br. Mus. (Nat. Hist.) Entomol. 58: 1–78.

Cox, J. M., and J. M. Wetton. 1988. Identification of the mealybug *Planococcus halli* Ezzat & McConnell (Hemiptera: Pseudococcidae) commonly occurring on yams (*Dioscorea* spp.) in Africa and the West Indies. Bull. Entomol. Res. 78: 561–571.

Ezzat, Y. M., and H. S. McConnell. 1956. A classification of the mealybug tribe Planococcini (Pseudococcidae: Homoptera). Bull. Md. Agric. Exp. Stn. A-e. 84: 1–108.

Kumar, S., K. Tamura, and M. Nei. 2001. MEGA: molecular evolutionary genetics analysis software for microcomputers. Comput. Appl. Biosci. 10: 189–191.

Rosen, D., and P. DeBach. 1977. Use of scale-insect parasites in Coccoidea systematics. Va. Polytech. Inst. & State Univ. Agric. Exp. Stn. Bull. 127: 5–21.

Rotundo, G., and E. Tremblay. 1975. Study of a sex pheromone of *Planococcus citri* (Risso) (Homoptera, Coccoidea). III. Specificity. Boll. Lab. Entomol. Agrar. Filippo Silvestri, Portici 32: 125–130.

Scheffer, S. J., and M. L. Lewis. 2001. Two nuclear genes confirm mitochondrial evidence of cryptic species within *Liriomyza huidobrensis* (Diptera: Agromyzidae). Ann. Entomol. Soc. Am. 94: 648–653.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651–701.

Swofford, D. L. 2003. PAUP* 4.0 beta 4a. Phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, MA.

Thompson J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876–82.

Thao, M. L., P. J. Gullan, and P. Baumann. 2002. Secondary (γ-Proteobacteria) endosymbionts infect the primary (γ-Proteobacteria) endosymbionts of mealybugs multiple times and coevolve with their Hosts. Appl. Environ. Microbiol. 68: 3190–3197.

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